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Evaluation of a New General Primer Pair for Rapid Detection and Differentiation of HSV-1, HSV-2, and VZV by Polymerase Chain Reaction

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The polymerase chain reaction (PCR) enables rapid and sensitive detection of VZV and HSV DNA and its efficiency depends mainly on the choice of the primers. Primers should hybridize to conserved DNA sequences within the viral genomes in order to avoid unreliable amplification due to DNA sequence variation between different strains. The aim of the study was to design and to evaluate a general primer pair which permits fast and reliable detection of HSV and VZV. The genes UL 15 of HSV and UL 42 of VZV share the highest degree of homology within the two genomes. We designed a primer pair (GPHV-RU) which hybridizes to these genes. The genetic variability of amplified sequences from clinical specimens was analyzed by restriction enzyme cleavage analysis and by temperature gradient SSCP analysis (TG-SSCP). PCR with GPHV-RU amplified viral sequences from all analyzed specimens (25xVZV, 10xHSV-1, 5xHSV-2) obtained from patients with clinical evidence of HSV or VZV infection. Restriction enzyme cleavage analysis with Hpa II further permitted reliable distinction between VZV, HSV-1, and HSV-2. Analysis of the heterogeneity of the amplified sequences by restriction enzyme cleavage and by TG-SSCP demonstrated no variability between the analyzed clinical specimens of VZV and of HSV-2 and only one differing TG-SSCP-pattern within the HSV-1 isolates. The results suggest that detection of HSV and VZV using the new primer pair GPHV-RU should give reliable results as the amplified sequences show little genetic variability within clinical isolates of HSV-1/2 and VZV.

KEY WORDS: temperature gradient SSCP, genetic variability, UL 15, UL 42

INTRODUCTION

Varicella-zoster virus (VZV) and herpes simplex virus (HSV) are important human pathogens and belong to the subfamily alpha herpesviruses. In general, the clinical diagnosis of herpes simplex, varicella, and zoster presents few difficulties once the eruption has fully developed. Nevertheless, virological confirmation is required in the case of zosteriform herpes simplex and in atypical VZV and HSV infections of immunosuppressed patients [Schimpff et al, 1972; Penneys et al., 1991]. Differential diagnosis of HSV and VZV infection may be achieved by viral culture, by Tzanck smear in combination with immunofluorescence or by the identification of viral DNA with the polymerase chain reaction [Hitchcock et al., 1974; Nahass et al., 1992]. The polymerase chain reaction (PCR) [Saiki et al., 1985] has the advantage of detecting minimal amounts of viral DNA even from small and poorly preserved samples such as crust from older lesions and stained and formalin-fixed tissue sections [Nahass et al., 1995]. PCR has also been suggested as the "gold standard" for the diagnosis of herpes simplex encephalitis from cerebrospinal fluid [Lakeman and Whiteley, 1995]. The specificity of the PCR process relies on the primers chosen which hybridize to the target DNA. A multitude of different primer pairs may be used for the detection of a given viral genome and many different primer pairs have been suggested for the diagnosis of HSV and VZV infections [Cao et al., 1989; Penneys et al., 1991; Shimizu et al., 1994]. Still, sensitivity and specificity may vary with different primer pairs and the genetic variability of naturally occuring strains of viruses is one important influencing factor. In general, the amount of genetic variability is not distributed evenly within the virus genomes and, therefore, primers should hybridize to more conserved DNA sequences in order to ensure reliable DNA amplification of naturally occuring variants.

Conserved DNA sequences may be identified by comparison of the DNA sequences of related viruses. The genes UL 15 of HSV and UL 42 of VZV share the highest degree of homology within the two genomes [Davison

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and Scott, 1986; McGeoch et al., 1988; Baines et al., 1994].

In order to obtain a PCR system which detects HSV as well as VZV and which is not influenced by sequence variation of naturally occuring variants, we designed the general primer pair (GPHV-RU) which hybridizes to these genes. PCR with GPHV-RU was evaluated for the clinical diagnosis of HSV and VZV infections and the genetic variability of the amplified DNA sequences was examined by restriction enzyme cleavage analysis and by temperature gradient single strand conformation polymorphism analysis (TG-SSCP) [Rübben et al., 1995].

MATERIAL AND METHODS

Clinical Specimens and Control DNA

Patients with the clinical diagnosis of VZV (n = 25) and HSV (n = 15) infection were entered into the study. Vesicles were wiped with an alcohol swab and opened with a 22-gauge-needle. The vesicle fluid was collected on a sterile cotton-tipped applicator. For the collection of crust-material the applicator was first moistened with sterile water. Vesicle fluid and crust material were suspended in 500 μl of sterile water and spun in a microfuge for 2 minutes. The supernatant was discarded and the pellet was resuspended with 10 μl of sterile water. Extracted DNA from VZV and typed serologically HSV type 1 and type 2 cultures served as positive control.

PCR Amplification

In order to amplify DNA of HSV and VZV we designed a new general primer pair (GPHV-RU) [sense primer 5′-TCCTGGCTGCTNTTTCCCTC-3′, antisense primer 5′-TGGCCGANTCCTTCNTGCAGGA-3′ (N = A/C/G/T)] after sequence alignments of the HSV 1 and VZV genomes. GPHV-RU hybridizes to the genes UL 15 of HSV and UL 42 of VZV. Amplification of CMV and EBV DNA was excluded by sequence analysis.

Each 50 μl reaction contained 2 μl of the suspended pellet, 10 pmol of each primer, 100 ng of each deoxynucleoside triphosphate, and 5 μl of standard PCR-buffer (10 mM Tris hydrochloride, 500 mM potassium chloride, 15 mM magnesium chloride; pH 8,3 at RT). The reaction mix was denatured for 5 minutes at 99°C in order to extract the viral DNA. 0.5 units of Tth DNA polymerase (Pharmacia) were added at 80°C and amplification was carried out with 35 cycles of 1 minute denaturation at 94°C, 1 minute annealing at 55°C, and 1 min extension at 72°C. Amplification terminated with an extension step of 5 min duration after the last cycle. PCR-products were separated on 1.0% agarose gels (1xTBE) and stained with ethidium bromide.

Restriction Enzyme Cleavage Analysis

Analysis of PCR-products by RFLP was carried out with the endonucleases Dde I (Pharmacia) and Hpa II (Pharmacia). 10 μl of each PCR-product were digested for 2 hours at 37°C with 1 unit of enzyme. The fragments were separated on 1.5% agarose gels.

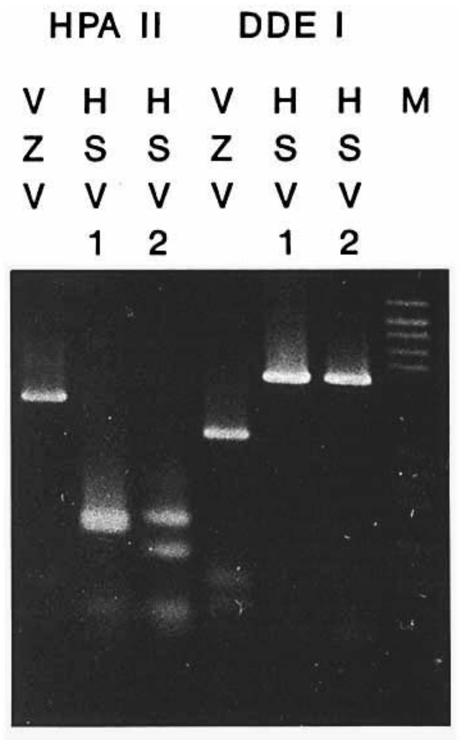

Fig. 1. Restriction enzyme cleavage patterns from the PCR products obtained with the control VZV, HSV-1, and HSV-2 DNAs after digestion with the endonucleases Dde I and Hpa II.

TG-SSCP Analysis

Temperature gradient single strand conformation analysis was undertaken as described previously [Rübben et al., 1995]. Briefly, 3.5 μl of each PCR-product were mixed with a stop solution containing 3 μl of 95% formamide, 10 mM NaOH, 0.05% bromophenol blue, and 0.05% xylene cyanol. The mixture was denatured at 100°C for 5 minutes, chilled rapidly on ice for 3 min, and then filled in a single well of a horizontal electrophoresis gel. Electrophoresis with a linear temperature gradient from the cathode to the anode ranging from 19°C to 25°C was carried out in a 0.5% Hydrolink gel matrix (AT Biochem, Malvern, PA) at 300 V for 3.5 hours. Nucleic acids were visualized by silver staining.

RESULTS

PCR with the primer pair GPHV-RU amplified a 396 basepair (bp) product with HSV-1 and HSV-2 control DNA and a 405 bp product with VZV control DNA as predicted by the published DNA sequences of HSV [McGeoch et al., 1988] and VZV [Davison and Scott, 1986]. Incubation of the PCR products with the restriction enzyme Dde I demonstrated DNA cleavage (fragments of 275 bp, 74 bp, and 56 bp) only with VZV DNA as expected by sequence analysis (Fig. 1). Cleavage with the Hpa II endonuclease of the PCR product generated with VZV DNA yielded the predicted fragments of 336 and 69 bp length and cleavage with Hpa II of the PCR product generated with the HSV-1 DNA demonstrated

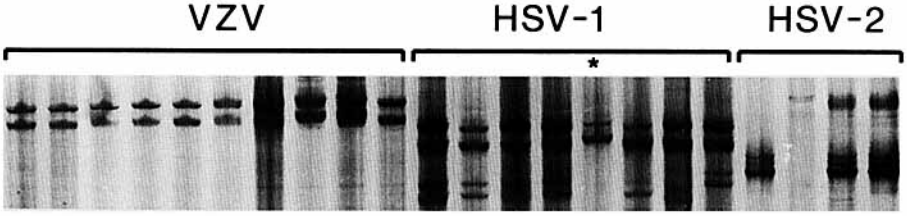

Fig. 2. TG-SSCP patterns generated with clinical specimens from 10 VZV, eight HSV-1, and three HSV-2 lesions. The asterisk indicates a variant TG-SSCP pattern within the HSV 1 samples.

the expected major fragments of 134 and 147 bp length. Endonuclease digestion with Hpa II of the amplification product obtained with HSV 2 control DNA showed predominant DNA bands at approximately 170 and 110 bp, thus enabling the differentiation of HSV-1 and HSV-2 (Fig. 1). PCR with the primer pair GPHV-RU amplified viral DNA sequences with all 40 specimens obtained from patients with clinical evidence of HSV or VZV infection. Restriction enzyme cleavage analysis with Dde I and Hpa II was carried out in order to distinguish between VZV, HSV-1, and HSV-2 and to analyze the genetic variability of the amplified DNA sequence from different clinical isolates. All 25 specimens obtained from VZV lesions displayed the same cleavage pattern as the control VZV DNA, 10 DNA templates from HSV lesions located on the face and trunk showed the fragments of HSV-1 and all 5 PCR products obtained from genital HSV lesions showed the HSV-2 restriction enzyme cleavage pattern. The genetic variability of the amplified sequences was analyzed further by temperature gradient single strand conformation polymorphism analysis. Using this method all VZV samples demonstrated the same band pattern. Figure 2 shows the TG-SSCP patterns obtained with 10 of the 25 analyzed specimens. From the 10 HSV type 1 DNAs only one specimen showed a shifted TG-SSCP pattern (Fig. 2, asterisk) and all TG-SSCP pattern generated with HSV-2 DNAs were equal. Figure 2 demonstrates the results obtained with eight of the 10 HSV-1 and 3 of the 5 HSV-2 specimens. Thus, TG-SSCP confirmed the absence of significant genetic variability within the amplified DNA sequence from different clinical specimens.

DISCUSSION

The aim of the study was the establishment of a robust PCR detection method for the identification of HSV and VZV DNA which would not be influenced by DNA sequence variation within naturally occuring isolates. Theoretical considerations suggest that DNA sequences with high homology between two virus species also display little variability within naturally occuring variants of each virus species. Therefore, the general primer pair GPHV-RU was designed which amplifies within the genes UL 15 of HSV and UL 42 of VZV which share the highest degree of homology. The correctness of this assumption was confirmed by the analysis of the genetic heterogeneity of the PCR products generated with the primer pair GPHV-RU from 40 clinical specimens. Restriction enzyme cleavage analysis with two endonucleases showed no variability within the VZV samples and only demonstrated the distinction between type 1 and type 2 of HSV. The PCR products were also analyzed by TG-SSCP analysis [Rübben et al., 1995] which is a modification of SSCP analysis [Orita et al., 1989] as restriction enzyme cleavage analysis only detects sequence changes affecting restriction enzyme sites. In SSCP, single stranded nucleic acid segments are separated in a nondenaturing polyacrylamide gel. Sequence mutations which alter the conformation of the single stranded DNA are detected by bandshifts. SSCP is very sensitive as it is able to demonstrate single nucleotide substitutions in homologous DNAs [Orita et al., 1989]. Temperature gradient SSCP was developed as the conformation of single stranded nucleic acid in SSCP analysis is temperature-dependent. The temperature gradient in the polyacrylamide gel enhances the possibility that one DNA segment adopts a conformation which differs from the homologous single stranded nucleic acid. Using TG-SSCP only one variant DNA amplification product was found within the analyzed HSV-1 samples, thus, TG-SSCP confirmed the results obtained by restriction enzyme cleavage analysis.

For routine application, PCR with the primer pair GPHV-RU and subsequent restriction enzyme cleavage with Hpa II is sufficient to detect and distinguish between VZV, HSV type 1 and HSV type 2 DNA. As the genetic variability of the amplified sequences seems to be minimal within the naturally occuring isolates examined, typing by restriction enzyme cleavage should be very reliable and no hybridization step should be required for confirmation.

The results of this study suggest therefore, that the presented PCR detection method for HSV and VZV which uses the general primers GPHV-RU should permit a reliable and straightforward molecular diagnosis of HSV and VZV infections.

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